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David Lukton
308-3213
AU 1653
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Mailbox room: 9B01
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AU Chen K; Lin Y; Detwiler T C
CS Department of Biochemistry, State University of New York Health Science
Center, Brooklyn 11203.
NC HL37250 (NHLBI)

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Protein Disulfide Isomerase Activity Is Released by Activated Platelets

By Kui Chen, Yin Lin, and Thomas C. Detwiler

The release of protein disulfide isomerase by activated platelets was hypothesized on the basis of reported intermolecular and intramolecular thiol-disulfide exchange and disulfide reduction involving released thrombospondin in the supernatant solution of activated platelets (Danishefsky, Alexander, Detwiler: *Biochemistry*, 23:4984, 1984; Speziale, Detwiler: *J Biol Chem*, 265:17859, 1990; Speziale, Detwiler: *Arch Biochem Biophys* 286:546, 1991). Protein disulfide isomerase activity, measured by catalysis of the renaturation of ribonuclease inactivated by randomization of disulfide bonds, was detected in the supernatant solution after platelet activation. The activity was inhibited by peptides known to inhibit protein disulfide isomerase; the peptides also

inhibited formation of disulfide-linked thrombospondin-thrombin complexes. The reaction catalyzed by the supernatant solution showed a pH dependence distinct from that of the uncatalyzed reaction. The activity was excluded by a 50-Kd dialysis membrane, and it was eluted in the void volume of a gel-filtration column, indicating that it was associated with a macromolecule. The activity was not removed by centrifugation at 100,000g for 150 minutes indicating that it was not associated with membrane microvesicles. Possible functions for the release of protein disulfide isomerase by activated platelets are discussed.

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Platelets are activated at sites of injury, and activated platelets release a variety of proteins and other substances that presumably play roles in the arrest of bleeding and in the tissue remodeling associated with wound healing. Based on our observations¹⁻⁶ that released thrombospondin (Tsp) undergoes intramolecular and intermolecular thiol-disulfide exchange and the reduction of one disulfide bond, we hypothesized the release of an enzyme to catalyze these reactions. Protein disulfide isomerase (PDI) (for reviews of PDI see refs 7 and 8) catalyzes the isomerization of disulfide bonds in proteins from one cyst-cys pairing to another,⁷ and it catalyzes the disulfide cross-linking of two polypeptide chains.^{9,10} PDI also exhibits protein thiol-disulfide oxidoreductase activity^{11,12}; it catalyzes either the oxidation of thiols to disulfide bonds or the reduction of disulfide bonds to thiols, depending on the relative redox potentials of the proteins and other thiol/disulfide pairs in the medium.

In this paper we report that a protein with PDI activity is released by activated platelets. The importance of this observation is that disulfide isomerization at the site of an injury raises many intriguing possibilities, including modification of the function of proteins by reduction or isomerization of disulfide bonds and the covalent cross-linking of proteins to each other and to cells.

MATERIALS AND METHODS

PDI assay. PDI activity was assayed by measurement of the renaturation of ribonuclease (RNase) that had been inactivated by formation of randomly mismatched, or "scrambled," disulfide bonds^{7,8,13}; such an RNase is referred to as scrambled RNase. In

the presence of a low concentration of a thiol reagent, scrambled RNase renatures slowly, regaining enzyme activity; PDI accelerates this renaturation. The renaturation can be measured by the action of the renatured RNase on RNA. We used the procedure of Hillson et al¹³ with the indicated modifications. The solution to be tested for PDI activity was incubated at 24°C with 5 µg/mL (instead of 50 µg/mL) scrambled RNase and 750 µmol/L reduced glutathione (instead of 10 µmol/L dithiothreitol) in a total volume of 100 µL. The extent of renaturation of scrambled RNase was determined by transferring 10 µL of the reaction mixture to 2.5 mL of a solution containing 80 µg/mL yeast RNA, 50 mmol/L Tris-HCl (pH 7.5), 25 mmol/L KCl, and 5 mmol/L MgCl₂ at 24°C. The rate of increase in A₂₆₀ of this solution was a measure of RNase activity. Each measurement included a control in which scrambled RNase was incubated in identical buffers (without any PDI activity) as a measure of the uncatalyzed renaturation.

Preparation and activation of platelets. Washed human platelets were obtained from blood of healthy volunteers by venipuncture. Blood was collected into 0.15 vol of anticoagulant containing 85 mmol/L sodium citrate, 65 mmol/L citric acid, and 1% glucose. Subsequent procedures were at 4°C. The blood was centrifuged at 300g for 20 minutes to obtain platelet-rich plasma, which was centrifuged at 1,000g for 20 minutes to sediment the platelets. The platelets were washed twice by resuspension in a solution containing 10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, and 1 mmol/L EDTA. A final suspension in this solution contained 1 to 3 × 10⁹ platelets/mL. Platelets were activated at 37°C by addition of 2.5 mmol/L CaCl₂ and 2.5 µmol/L calcium ionophore A23187 to the suspension. After 2.5 minutes, the suspension was centrifuged at 39,000g for 30 minutes to obtain the supernatant solution of activated platelets.

Thrombin. Human α-thrombin was a generous gift from John W. Fenton II (Wadsworth Center for Laboratories and Research, School of Public Health Sciences, New York Department of Health, Albany). The iodination of thrombin and the electrophoretic analysis of its complexes with Tsp have been described.²

Other reagents. A23187 was purchased from Calbiochem (San Diego, CA). Antithrombin III, somatostatin, and tocinoic acid were purchased from Sigma (St Louis, MO). Bacitracin A, prepared from bacitracin by chromatography, was a generous gift of Leo Kesner (Department of Biochemistry, SUNY Health Science Center at Brooklyn).

RESULTS AND DISCUSSION

Acceleration of the rate of renaturation of scrambled RNase by the supernatant solution of A23187-activated platelets is shown in Fig 1A. Activity in the supernatant solution before activation of the platelets was negligible if

From the Department of Biochemistry, State University of New York Health Science Center at Brooklyn, Brooklyn, NY.

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Address reprint requests to Thomas C. Detwiler, PhD, Department of Biochemistry, SUNY HSCB, Brooklyn, NY 11203.

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spontaneous activation was avoided by inclusion of 2.5 $\mu\text{mol/L}$ prostaglandin E_1 (PGE_1). Sixty percent of the activity was lost at 100°C (Fig 1A, triangles). The activity was inhibited by three peptides known to inhibit endoplasmic reticulum PDI¹⁴⁻¹⁶ (Fig 1B). The catalyzed renaturation showed a pH dependence distinct from the uncatalyzed renaturation (Fig 2). The activity was not dialyzable through a 50-Kd cutoff membrane, and it was eluted from a Sephadex G-200 (Pharmacia-LKB, Piscataway, NJ) column in the void volume (data not shown), both suggesting that it was associated with a large macromolecule. We conclude that activated platelets release a macromolecule, presumably a protein, with PDI activity.

We hypothesized the release of PDI from platelets on the basis of three phenomena observed with Tsp in the supernatant solution of activated platelets. First, complexes of thrombin with certain serpins become disulfide linked to Tsp^{1,2} and Tsp becomes disulfide linked into multimers⁶ by processes that require free thiols. The most likely mechanism is thiol-disulfide exchange leading to a new intermolecular disulfide bond. Second, Tsp has one equivalent of thiol per polypeptide chain, but at least 12 different cysteinyl residues were labeled with a thiol-reactive reagent,⁴ indicating that there was isomerization of disulfide bond pairings. This is likely due to intramolecular thiol-disulfide exchange. Third, incubation of the supernatant solution led to an increase of nearly two thiols per mole of Tsp,⁵ indicating reduction of a disulfide bond. Because each of these three reactions is typical of one catalyzed by PDI,^{7,8} we tested the effect of bacitracin A, a PDI inhibitor¹⁴⁻¹⁶ (Fig 1B), on one of them, the disulfide bonding of Tsp to thrombin-ATIII complexes. (In unpublished experiments by A.C. Chang and T.C. Detwiler, it has been shown that thrombin becomes disulfide linked to Tsp when it is complexed with ATIII as well as when it is complexed with released platelet protease nexin. The reactions require the supernatant

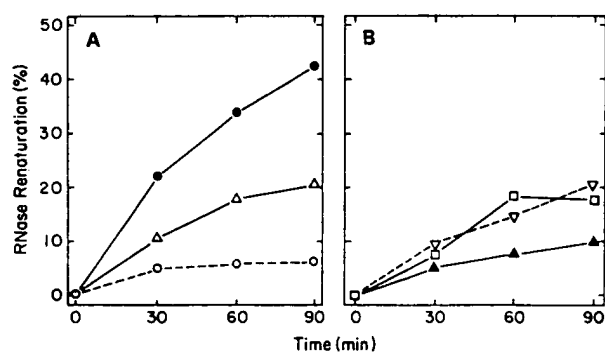


Fig 1. Renaturation of scrambled RNase by incubation with the supernatant solution of activated platelets. The renaturation of scrambled RNase was measured as described in Materials and Methods. The RNase activity is presented as a percent of the activity if it were completely renatured. (A) Scrambled RNase was incubated in (○---○), suspension buffer; (—), supernatant solution of activated platelets; or (△---△), supernatant solution heated in boiling water twice for 5 minutes each time. (B) The same supernatant solution was incubated with scrambled RNase in the presence of (▲---▲), 3.5 mmol-L bacitracin A; (▽---▽), 200 $\mu\text{mol/L}$ somatostatin; or (□---□), 500 $\mu\text{mol/L}$ tocinoic acid.

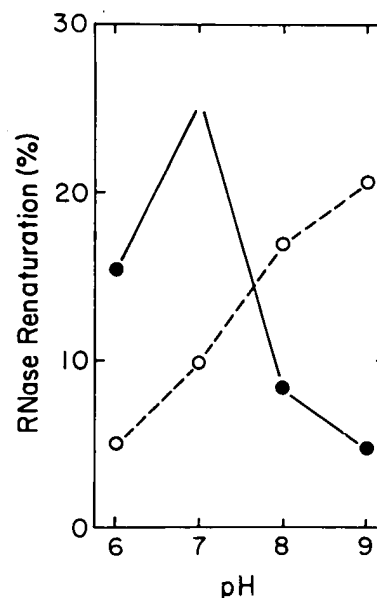


Fig 2. The effect of pH on the renaturation of scrambled RNase in the supernatant solution of activated platelets. The supernatant solution (●—●) or the suspension buffer (○---○) was adjusted to the indicated pH before incubation with scrambled RNase. For this experiment the reductant was 10 $\mu\text{mol/L}$ dithiothreitol instead of glutathione. The activity in the supernatant solution has been corrected for the spontaneous renaturation in the buffer alone.

solution.) Formation of the complexes was inhibited by bacitracin A (Fig 3). This suggests that PDI is responsible for the reaction, but this must be qualified by the fact that the mechanism by which bacitracin A inhibits PDI is unknown. A more definitive test will require a pure enzyme or specific antibodies.

The best-studied protein disulfide isomerase is membrane associated^{7,8} as an enzyme of the endoplasmic reticulum, where it functions in post translational modification of proteins. Because A23187-activated platelets are known to release membrane microvesicles,^{17,18} we asked whether the released PDI activity was membrane associated. However, the activity in the supernatant solution was not removed by centrifugation at 100,000g for 150 minutes, a condition that removes platelet microvesicles. There are proteins with PDI activity that are not associated with membranes; they include thioredoxin^{19,20} and the peptide hormones lutropin and follitropin.²¹

One can imagine many possible roles for PDI at a site of injury, where platelets are activated. It was proposed, for example, that the intramolecular isomerization of disulfide bonds of thrombospondin reflected a change in conformation when Tsp went from the α -granule to the extracellular medium.⁴ In this regard, the preliminary report²² that the cell adhesive activity of thrombospondin depends on the state of disulfide bonds is especially interesting; it suggests that the cell adhesive properties of Tsp might depend on PDI. PDI might also catalyze the covalent cross-linking of matrix proteins or the covalent immobilization of biologically active molecules to the extracellular matrix.

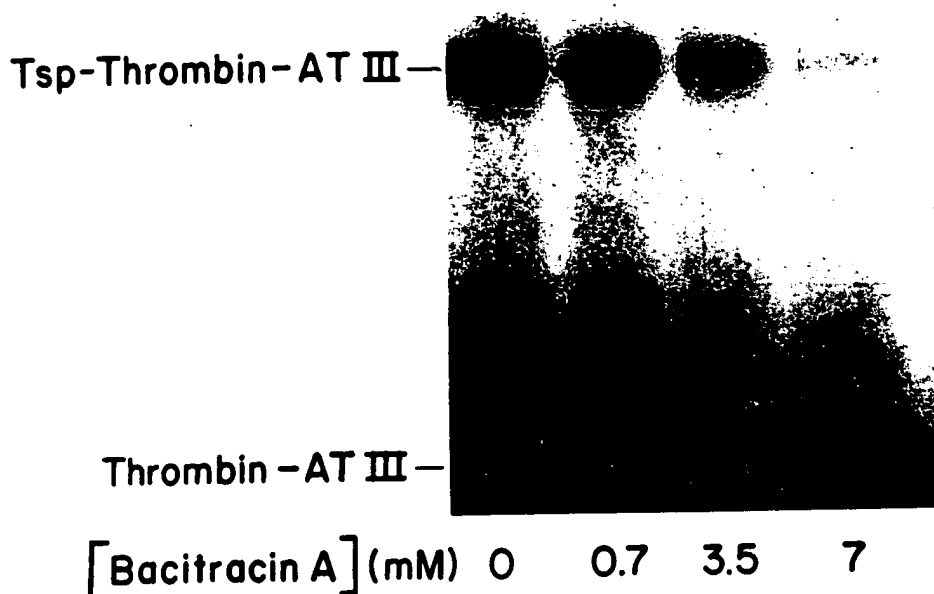


Fig 3. The effect of bacitracin A, a PDI inhibitor, on formation of disulfide-linked complexes of Tsp with thrombin-ATIII. Two microliters of 5 nmol-L ¹²⁵I-labeled thrombin was incubated with 8 μ L of 50 U/ml ATIII for 10 minutes at room temperature. Ninety microliters of an activated supernatant solution containing the indicated concentrations of bacitracin A was added and incubated for 20 minutes at 37°C. The sample was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/autoradiography as described.¹⁻³

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